A Study on Identification and Characterization of Protein Biomarkers in Saliva of Schizophrenia Subjects

A

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INTRODUCTION

According to the world mental health report 2001, approximately 24 million people worldwide are suffering from schizophrenia. The incident rate of schizophrenia is believed to be about 0.5 per 1000 **(Ahuja, 2006).** The annual incident rate of schizophrenia is higher in rural than urban population e.g. analytical values are 4.4/1000 in rural and 3.8/1000 in urban population **(Wig *et al*., 1993).** The prevalence of schizophrenia is approximately 0.5–1%. Schizophrenia is prevalent across racial, sociocultural and national boundaries, with a few exceptions in the prevalence rate in some isolated communities **(Ahuja, 2006).** A research with 188 studies across 46 countries for the period of 1965 to 2002, estimated the median value of point prevalence at 4.6 per 1000 person and for lifetime prevalence at 7.2 per 1000 **(Saha *et al*., 2005; Gelder *et al*., 2004).**

The interference in brain development during early life, brain infection, neurodevelopmental and abnormalities in early brain development, generate some type of neurodegenerative diseases **(Khandaker *et al*., 2012).** However, schizophrenia originates from dysfunction of neurotransmission which contributes genesis of psychotic disturbance of synaptic function leading to abnormalities of neuronal connectivity in interneuron **(Owen *et al*., 2016).** Schizophrenia literally means as "*split of mind*" i.e. change in nature, emotions, mood and behaviour of the sufferer **(Picchioni and Murray, 2007**). It is most puzzling yet disabling of brain disease with its severe and persistent psychiatric manifestation accompanied by variable cognitive dysfunction and profound psychosocial impairment **(Ruiz *et al*., 2000).** Thus the researchers believed that some symptoms represent a relatively pure loss of function (negative symptoms answer to the dissolution), while positive symptoms such as delusion and hallucination represented an exaggeration of normal function **(Gelder *et al*., 2004; Mos, 2015).** There is two syndrome hypothesis of schizophrenia i.e. type-I schizophrenia is characterized by prominent positive symptoms and type-II schizophrenia is characterised by prominent negative symptoms **(Gelder *et al*., 2004)**. Some of the main symptoms that help in detection of schizophrenia patients are as following; [**I] Positive symptoms- (1) Delusion**- false unshakable belief, delusion of reference (e.g. people are talking about me), delusion of persecution (e.g. people are against me), delusion of grandeur (e.g. I am God almighty), delusion of control (e.g. my

neighbours are controlling me) and somatic (e.g. there are insects crawling in my scalp); **(2) Hallucination–** it occurs in hearing, smell, touch, and vision about what was not actually there, auditory hallucinations are by far the most frequent; **(3) Disorder of affect-** apathy, decreased emotional expression, blunting of emotions or no emotions, and inappropriate expression; **(4) Disorder of Motor Behaviour-** increased psychomotor activity (excitation, aggressiveness, restlessness and agitation); **[II] Negative symptoms**- the prominent **negative symptoms** of schizophrenia include affecting flattering or blunting, attention impairment, alogia (lack of speech output), associality (social withdrawal. The other features could be decreased functioning in work and loss of ego boundaries **(Picchioni and Murray, 2007, Gelder *et al*., 2004, Ruiz *et al*., 2000; Ahuja 2006).** Thought disorder (TD) in which lack of somatic health care and social functioning impairment generate a negative impact on schizophrenia patients, leads to hyposecretion of saliva **(Mos, 2015).** The occurrence of schizophrenia in men and women leads the difficulty in married life, chronic illness, job obligations and difficulty in pregnancy and childbirth in women **(Loganathan and Murthy, 2011).** Whereas, the bipolar disorder associated with severe disability in lifetime differentiate in two categories of manic episode which include patients feel very up, high, lot of energetic and increased activity levels while the other is depressive episode which include the patients feel very sad, down, empty and have decreased activity level and increased risk of committing suicide **(Iavarone *et al.*, 2014).** Psychiatric co-morbidities are most common in patients of schizophrenia and also contain symptoms of anxiety leading to depressive symptoms and addiction of drugs and alcohols. The prevalence rate of anxiety disorder gets increased in schizophrenia patients **(Buckley *et al.*, 2008).** Depressive symptoms in schizophrenia patients are related to long duration of hospitalization, poor response to medicines, improper social and cognitive functions, and higher rates of relapse. In addition, the depressive condition is one of predictor of human suicide (approx. 60%) in schizophrenia **(Noto *et al.*, 2011).**

The executive functions include the capacity to formulate goals, plan and organise goal directed behaviour carry out goal directed behaviour fully and effectively monitor self-correct ones behaviour as needed by using direct and indirect clinical neuropsychologically by various test and their symptom patterns were quantified using the Positive and Negative Syndrome Scale (PANSS). The longitudinal

studies assess the impact of cognitive function and disability in patients with schizophrenia. It was seen that the literature on self-care suggests that predictive power of cognitive deficits in determining the trajectory of functional abilities **(Rajji *et al.,* 2013; Sabhesan and Parthsarthy, 2005)**. Schizophrenia disorder is diagnosed on the basis of clinical investigations by expert and by criteria of diagnosis statistical manual of mental disorder. The six criteria (A–F) for the diagnosis of schizophrenia in DSM-IV will be retained with modest DSM-5 which provide a more useful platform than the current DSM-IV in integrating emerging genetic and other neurobiological information about these conditions **(Ruiz *et al.,* 2000; Tandon *et al.,* 2013).** Treatment of schizophrenia is done as (a) somatic treatment, which includes pharmacological treatment, electro-convulsive therapy (ECT) and miscellaneous treatment, while (b) psychosocial treatment includes rehabilitation **(Ahuja 2006).**

On an average, a human being produces 1.0 to 1.5 litres of saliva every day. The saliva of humans contains approximately more than 2000 proteins, which are almost 27% of the total blood proteins **(Podzimek *et al.,* 2016; Javaid *et al.,* 2016).** These proteins are entering into saliva from the blood with the help of the gaps between the cells. Depletion in the saliva secretion in humans creates some problem like painful tongue and mucosa, tooth loss, tooth decay, problem with taste, chewing, swallowing and phonation and increased risk for infection **(Schenkels *et al.,* 1995; Mandel., 1987).** Calcium ions, potassium ions, phosphorus ions and chloride ions are main constituent in the formation of saliva **(Rehak *et al.,* 2000).**

Human saliva contains proteins that can be informative for disease detection and surveillance of brain health. Comprehensive analysis and identification of the proteomic content in whole saliva is a necessary first step towards the discovery of salivary protein marker(s) for human brain disease detection. Quantitative proteomics approach leads to develop discriminatory biomarker signatures from human saliva for detection of schizophrenia disease **(Nascimento and Martin-de-souza, 2015).** In schizophrenia, protein biomarkers have potential due to their large diversity, hundreds of proteins detected with high sensitivity in saliva, blood serum and cerebrospinal fluid (CSF) **(Amorin *et al.,* 2017).** Specific biomarker protein which is found in saliva such as Tau protein, belongs to family of microtubule associated protein and was the first biomarker for Alzheimer's disease **(Buee *et al.,* 2000).**

The measurement of salivary alpha-amylase level might be one of the useful biological markers for assessment of psychotic state and efficiency of treatment in patients of schizophrenia **(Ieda *et al.*, 2012).** Salivary cortisol has also been found as biomarker of physiological stress **(Hellhammer *et al.*, 2009).** Inflammation markers in saliva are increased in acute stress due to social-cognitive and physical exercise **(Slavish *et al*., 2015).** A 2-D polyacrylamide gel electrophoresis (2D PAGE) coupled with mass spectrometry (MS) are used in a number of studies for detection and identification of human saliva proteins, and thus called as 2D-PAGE-MS, which lead to approximately thousands of proteins being identified in whole saliva **(Hu *et al.*, 2007**). Single nucleotide polymorphisms (SNPs) can result in protein-sequence polymorphisms (PSPs) when codon translations are altered. Both top-down and bottom-up proteomics strategies can identify PSPs, and a 14,319 Da protein from human saliva was characterized using the top-down approach on a hybrid linear ion-trap Fourier-transform ion cyclotron resonance mass spectrometer **(Whitelegge *et al.*, 2007).** The salivary alpha-amylase has been considered as a useful tool in evaluating schizophrenia and association between salivary alpha-amylase change and psychiatric state is found in schizophrenia patients **(Inagaki *et al*., 2010).** The protein DJ-1 is found in human saliva and acts as potential biomarker in Parkinson's disease (**Master *et al.*, 2015).** Nerve growth factor (NGF), later called as brain-derived neurotrophic factor (BDNF) is a second member of the "neurotrophin" family of neurotrophic factors **(Binder and Scharfman, 2004**) and it act as a best biomarker for neurodegenerative disorder and the up-regulation and down-regulation of BDNF levels in human saliva is indicator of neurodegenerative diseases like Alzheimer's disease, schizophrenia disorder, Parkinson's disease, and motor neurons disease **(Baily, 1996).** In Alzheimer's disease, the BDNF level is decreased **(Phillips *et al.*, 1991)** and serum BDNF levels also gets decreased in untreated major depression patients **(Mercader *et al*., 2008; karege *et al*., 2002).** Down-regulation and abnormalities in BDNF factor or signalling in the brain, has been implicated as biomarker in schizophrenia and mood disorders **(Ray *et al*., 2014).** Some proteins are found in human saliva such as histamine, proline-rich protein (PRPs), lysozyme, immunoglobin-A, alpha-amylase and a specific protein kallikrein is secreted by mucosal glands and is present in saliva **(Schenkels *et al.*, 1995).** The genetic study of schizophrenia has indicated that the mode of transmission is complex. The number of

susceptibility loci, the disease risk conferred by each locus, the extent of genetic heterogeneity, and the degree of interaction among loci all remain unknown **(Craddock *et al*., 2005).**

Schizophrenia and bipolar disorder are two different mental health disorders. Schizophrenia causes symptoms that are more severe than the symptoms of bipolar disorder. A person with bipolar disorder uses to switch between extreme excitement, mania or depression and also, to some extent, may experience hallucinations (seeing or hearing things that are not there) and delusions (beliefs in things that are not true). Having being more severe condition, the schizophrenia patients undergo extreme case of hallucinations and delusions, along with social isolation, moodiness, loss of interest in activities and violent outbursts etc. Such subjects do also experience disorganized thinking in which they are unable to care for themselves.

As per a study of **Iavarone *et al,* 2014**, the symptoms of schizophrenia and bipolar disorder are different from each other. The proteomic profile of both the disorders increase in some common salivary proteins levels with respect to healthy control and no difference was observed in proteins levels between schizophrenia and bipolar disorder; however the authors left a caveat to further evaluate the sensitivity and specificity of the differentially expressed proteins.

We shall be incorporating the help of executive functions of schizophrenia subjects such as regulation of goal-directed formulation, behavioural programme, working memory and effective performance by using various psychological tests. The high throughout mass spectrometry (MS/MS) based proteomics shall be used to identify, quantify, interaction and validation of proteins in saliva of subjects of schizophrenia verses healthy controls, which may act as potential biomarkers of disease and to better understand the proteomics of schizophrenia disorder.

REVIEW OF LITERATURE

To understand the previous work done by different researchers, we have studied the relevant literature, so that we can glance over the work already been done and what to be done further and also about following the protocols accordingly.

Mandel (1987) studied the important functions of saliva like anti-bacterial, anti-fungal, anti-viral activity, regulation of pH, maintenance of tooth, mechanical clearing and water balance in human body. **Wig *et al.* (1993)** studied the incident study of schizophrenia in India and actively identified 268 first onset potentially schizophrenia cases and find out annual incident rate as 4.4/1000 in rural areas & 3.8/1000 in urban areas. **Lenzenweger and Korfin (1994)** studied the Wisconsin card sorting test (WCST) by 23 schizotypy subjects and 28 normal control subjects overall schizotypy perceptual Aberration Scale Subjects (high PAS) performed more poorly than control (low PAS) subjects on WCST. **Schenkel *et al.* (1995)** studied the biochemical composition of human saliva like histamins that act as antifungal and antibacterial, mucins which helps in visco-elasticity, alpha-amylase help in digestion and relation to other mucosal fluids and their distribution in other mucosal secretions. **Baily (1996)** studied the nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and state that brain-derived neurotrophic factor is similar to nerve growth factor. **Rehak *et al.* (2000)** investigated the electrolyte balance and biochemical composition in unstimulated saliva by method of saliva and serum concentration ratio of whole human saliva in healthy adult subjects. **Ruiz (2000)** detailed about clinical features, symptoms, diagnostic criteria, prevalence, agitation and hostility of schizophrenia in chapter of schizophrenia and other psychiatry disease of comprehensive textbook of psychiatry. **Binder and Scharfman (2004**) evaluated the nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) as another member of neurotropic factor which is found in saliva and help in survival of dorsal root ganglion neurons. **Gelder *et al.*, (2004)** studied the history, clinical features, diagnosis, classification, epidemiology and prevalence rate of schizophrenia in chapter of schizophrenia and acute transient psychotic disorder of new oxford textbook of psychiatry. **Craddock *et al.* (2005**) studied the schizophrenia and bipolar disorder genetics and dissecting psychosis and identify the established linkage genes in schizophrenia and bipolar disorder. **Lequin (2005)** studied the brief note addresses the

historical background of the invention of the enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA). **Sabhesan and Parthsarthy (2005)** evaluated the executive functions include the capacity to formulate goals, plan and organise goal directed behaviour carry out goal directed behaviour fully and effectively monitor self-correct ones behaviour needed. **Saha *et al.* (2006)** studied a systematic review of 188 studies in 46 countries published between 1965 and 2002 and estimated the median value of point prevalence. **Ahuja (2006)** describe about definition, their types, clinical feature, symptoms, diagnostic criteria, and aetiology of schizophrenia in chapter 5 of a short textbook of psychiatry. **Hu *et al.* (2007)** evaluated the proteomic content of whole human saliva which is useful in disease diagnosis and prognosis because saliva testing is very simple, non-invasive, safe and low cost. **Picchioni and murray (2007**) studied about schizophrenia, its cause, prevalence rate, diagnosis, prognosis and treatment of schizophrenia disease. **Whitelegge (2007)** investigated the single nucleotide polymorphism (SNPs) can result in protein sequence polymorphism (PNPs) when codon translations are altered and post translation modification in proteins from human saliva using top-down Fourier transform ion cyclotron resonance mass spectrometry. **Buckley *et al.* (2008)** studied the psychiatric comorbidities, anxiety and depression symptoms are very common among patients of Schizophrenia. **Hellhammer *et al.* (2009)** studied that salivary cortisol acts as potential biomarker in stress patients and measure the hypothalamus pituitary adrenal axis (HPAA) reactivity of receptor and modulator of hippocampus, hypothalamus and adrenal. **Inagaki *et al.* (2010)** investigated the increased level of alpha-amylase in saliva of schizophrenia patients and find out increased parasympathetic nervous activity and decreased sympathetic activity in schizophrenia patients. **Loganathan and Murthy (2011)** studied the interview based study of 200 schizophrenia patients of rural and urban clinics and explored the issue of sociocultural related stigma among suffering patients. **Noto *et al.* (2011)** evaluated that brain-derived neurotrophic factor acts as biomarkers in both schizophrenia and depressive patients and this disorder contain low level of brain-derived neurotropic factor. **Ambatipudi *et al.* (2012)** studied the quantitative proteomics of parotid saliva in primary Sjogren's syndrome and identified that out of 1246 proteins in which 529 proteins were variable in subjects and in controls. **Idea *et al.* (2012)** investigated the salivary alpha-amylase level which acts as biomarker tool for analysis of schizophrenia severity, evolution of therapy and considered as alpha-amylase level is higher in

schizophrenia patients as compared to healthy controls. **Khandeker *et al.* (2012)** investigated the childhood infection and adult schizophrenia with a meta-analysis of population based studies. **Rajji *et al.*, (2013)** studied the remained longitudinal studies assessing the impact of cognitive function and disability in patients with schizophrenia. It was seen that the literature on self-care suggests that predictive power of cognitive deficits in determining the trajectory of functional abilities. **Tandon *et al.* (2013)** studied the definition and description of schizophrenia in DSM-5 and analysed the changing in definition of DSM-4 schizophrenia is radially diagnosed, has fair validation and conveys useful clinical information therefore the essence of broad DSM-4 definition of schizophrenia is retained in DSM-5. **Iavarone *et al.* (2014)** studied the characterisation of different proteins like alpha-defensive, S100A12, cystatin-A, and systatin-B are increased in saliva of bipolar disorder and schizophrenia patients by the method of top-down proteomics. **Ray *et al.* (2014)** investigated the decreased brain-derived neurotrophic factor and TrkB mRNA expression in multiple cortical areas of patients in schizophrenia and mood disorders. **Master *et al.* (2015)** investigated the salivary DJ-1 protein as a potential marker in disease severity and their elevated level in Parkinson's disease. **Mahmood and Yang (2015)** described western blot technique by which researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. **Mos (2015)** studied the saliva secretion disorder in a schizophrenic patient, which is a problem in dental and psychiatric treatment and manifestation of hyposecretion and hyper secretion of saliva. **Nascimento and Martins-de-souze (2015)** studied the mass spectrometry based proteomics that identified and quantified variety of different proteins that act as biomarker to diagnosis, prognosis of schizophrenia disease. **Slavish *et al.* (2015)** investigated the salivary markers of inflammation in response to acute stress. **Javaid *et al.* (2016)** studied the saliva as a diagnostic tool for oral and systemic diseases that evaluated in reducing disease severity, complication and also increase the success rate therapy. **Podzimek *et al.* (2016)** evaluated that the salivary proteins act as biomarker of periodontal and general disease. **Owen *et al.* (2016)** studied the diagnosis, differential diagnosis clinical presentation, and signs and symptoms of schizophrenia. **Viacqua *et al.* (2016)** investigated the oligomeric alpha-synuclein is decreased in Parkinson's disease by using specific anti-α-synuclein and anti-oligomeric α-syn ELISA kit. **Amorin *et al.* (2017)** studied the consolidation of that information which provides a large-scale application network of schizophrenia biomarkers.

Yamuna and Mathu (2017) evaluated the different types of saliva like stimulated saliva and un-stimulated saliva and different methods of collection of saliva collection. **Giri *et al.* (2018)** investigated the 646 salivary proteins were identified, of which 57 proteins were present in ovarian cancer and breast cancer or both.

Different researchers have reported about various neurodegenerative diseases being detected by saliva biomarkers. The present work focuses on executive functions of subjects and to find out the characterisation and identification of protein biomarkers in saliva of schizophrenia subjects.

OBJECTIVES

1. To select the schizophrenia subjects by using diagnostic manual of mental disorder (as per DSM-5 criteria) and studying their executive functions.
2. Isolation and basic analysis of salivary proteins in pools of subjects and healthy controls through SDS-PAGE.
3. Determination of differentially expressed proteins in pools of subjects and healthy controls by mass spectrometry (MS/MS).
4. Analysis, validation and interpretation of proteomic results and predict out highly significant protein biomarker(s) of schizophrenia disease.

MATERIALS AND METHODS

Sample size

The sample size has been decided as following four pools in schizophrenia subjects and one pool in healthy control**.**

Schizophrenia subjects:

		Age	Number
(I)	**Rural Patients**–	18 – 35 years	10
		36 – 55 years	10
(II)	**Urban Patients–**	18 – 35 years	10
		36 – 55 years	10
	Healthy control -		20
	Total samples -		**60**

Inclusive and Exclusive criteria of subjects and control

Inclusion criteria –

- Schizophrenia disease shall be confirmed with the help of a psychiatric expert by using Diagnostic and Statistical Manual of Mental Disorder, 5th Edition (DSM-5). This step shall be followed at the Institute of Mental Health and Hospital, Agra.
- The age of subjects shall be between 18 to 55 years.
- Schizophrenia disease has been diagnosed and treatment has continuously been given to the subjects for at least last 2 years to the subjects.
- Subjects (and/or his/her family members) have given the written consent for saliva collection.

Exclusion criteria –

- Schizophrenia patients suffering from any other mental illness.
- Comorbid psychiatric illness or mental retardation.
- Comorbid substance abuse or dependence, except for nicotine (hence smokers could be considered for the saliva sample collection).

1. About the Institute of mental health and hospital, Agra.

The Institute of Mental Health and Hospital, Agra, was established on 9th September 1859. This is an autonomous institution under aegis of the Government of Uttar Pradesh. The institute is a tertiary reformer centre and post-graduate teaching hospital, which imparts training in discipline of psychiatry, clinical psychology, psychiatric social work and psychiatric nursing. The hospital serves a wide attachment area, which include the state of Uttar Pradesh, Madhya Pradesh, Rajasthan, Uttarakhand, Haryana and Delhi. It is spread over an extensive and beautiful campus of 172.8 acres land and is well known centre of treatment, training and research on mental disorders. The institute has sanctioned bed strength of 838 patients (623 male patients and 215 female patients). Hundreds of patients suffering from schizophrenia visit the institute from various places of northern India. Out of 300-350 mental cases visiting each day, approximately 25-30 new cases of schizophrenia are registered in the out patients department (OPD) of the institute.

METHODOLOGY

Executive function in schizophrenia

Executive functions of schizophrenia are performed by various psychological tests e.g. Wisconsin Card Sorting Test (WCST) & Positive and Negative Syndrome Scale (PANSS).

1. **Wisconsin card sorting test (WCST):** The WCST is a measure of executive function requiring the ability to develop and maintain an appropriate problem solving strategy. WCST consist of 128 response cards. There are four stimulus cards each having variation of some parameters, e.g. colour, form and number in four different ways. Forms of cards are represented by some shapes e.g. cross, circle, triangle and star shaped in colour of Red, Blue, Yellow and Green. A number of stimulus cards are represented to a participant to match the cards. The participants is being told about a particular match is right or wrong with original WCST cards and would consist of experimenter on one side of the desk facing and participant on the other side. The test would take approximately 1 hour to carry out and generates a number of psychometric score. WCST test is carried out on each subject in a quiet room, with minimal disturbance. (**Annexure – I) (Lenzenweger and Korfine, 1994)**.

2. **The Positive and Negative Syndrome Scale (PANSS):** The positive and negative syndrome scale is a medical scale used for measuring symptom severity of patients with schizophrenia. To assess a patient using PANSS, an approximately 45 minute clinical interview is conducted. The patients are rated between 1 to 7 on 30 different symptoms based on interview as well as reports from family members or primary care hospital worker. Positive scale contains 7 items, negative syndrome scale contains 7 items and general psychopathology scale contains 16 items **(Annexure- II)**. PANSS total minimum score is 30 and maximum score is 210. As 1, rather than 0, is given as the lowest score for each item, a patient cannot score less than 30 for the total PANSS score. Score are often given separately for positive items and negative items and general psychopathology items **(Sabhesan and Parthsarthy 2005)**.

Collection of saliva

The amount of saliva secretion varies between 800-1500 ml/day or 1.0-3.0 ml/minute with pH range of ~7.0 for unstimulated saliva. In salivary diagnostics, unstimulated saliva is often preferred to the stimulated whole saliva. For this research study, unstimulated whole saliva will be collect from rural and urban and age wise patient cohorts. In swabbing method, synthetic gauze sponge or cotton pad is introduced into the mouth at the orifices of major salivary glands. The subjects would be asked to chew such that the cotton pad gets soaked with the saliva. Saliva soaked sponge is removed and placed in a sterilized tube **(Yamuna and Muthu 2017; Vivacqua *et al.*, 2016).**

Addition of Protease Inhibitor

Immediately after the collection, appropriate volume of protease inhibitor cocktail would be added to the saliva samples, to prevent the degradation of protein. Sodium fluoride and sodium orthovanadate will also be added in samples to prevent protein digestion from protease phosphatase **(Giri *et al.*, 2018)**.

Handling of saliva after collection and Protein extraction

Unstimulated whole saliva will be collected at particular time in sterilized tubes. After collection of saliva, it is immediately preserved at appropriate temperature of -20^{0}C for further processing**.** Saliva samples would be thawed and centrifuged at 4^{0}C to separate supernatant (proteins), and protein concentration will determined by the Bradford method or BCA protein assay. Proteins will be precipitated with ethanol at 4^{0}C for overnight; the protein pellets will be dissolved in Urea/Tris or ammonium bicarbonate buffer accordingly **(Giri *et al*., 2018 and Podzimek *et al*., 2016).**

Protein processing with SDS-PAGE and trypsin digestion

SDS-PAGE shall be performed for getting basic protein profile and processing for in-gel trypsin digestion. Alternatively, whole protein may also be used for in-solution trypsin digestion. Sequencing specific trypsin protease will be utilized to cleave proteins into smaller fragments, or peptides to cleave proteins into smaller fragments, or peptides. The trypsin is one of best choice of protein digestion. Reduction and alkylation shall be performed sequentially with 10 mM dithiothreiotol (DTT) at 60°C followed by 50 mM iodoacetamide (IAA) at room temperature **(Giri *et al.*, 2018 and Ambatipudi *et al*., 2012).**

Protein identification by Mass Spectrometry

There shall be either in-gel digestion through cutting bands of SDS-PAGE protein or in-solution trypsin digestion of total protein, followed by mass spectrometry (MS). Two primary methods will be used for ionisation of proteins as either MALDI-TOF (matrix-assisted laser desorption/ionization- time of flight) or ESI-MS (electrospray ionization mass spectrometry) as per the availability. After mass spectrometry, m/z data of PMF (peptide mass fingerprinting) shall be processed on Mascot search engine or other relevant proteomics database of bioinformatics **(Giri *et al*., 2018, Sivadasan *et al,* 2015, & Nascimento and Martin-de-souza, 2015).**

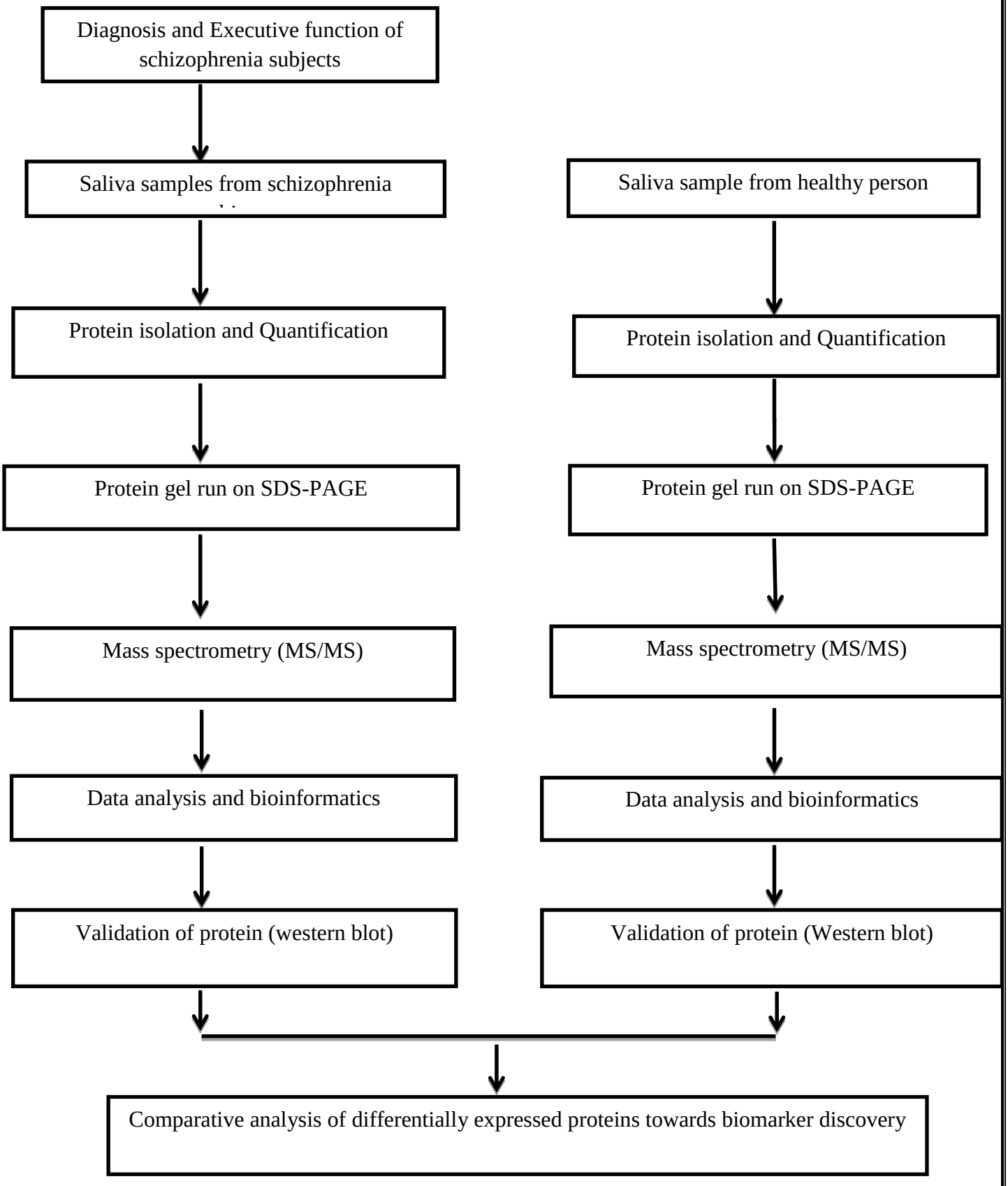
Data analysis

The identification of peptides from acquired MS/MS spectra will be performed using database search approach either by mascot search engine or through proteome discover 2.2 (thermo fisher scientific) as per our MS/MS data shall be performed by a mass spectrometry platform. The proteome search engines e.g. Uniprot and PDB (protein data bank) etc related to human protein database shall be thoroughly studied to compare the schizophrenia associated proteins in disease subjects and control groups **(Giri *et al*., 2018 & Sivadasan *et al*, 2015)**.

Western blot

Differential expression of highly significant selected proteins would be confirmed in schizophrenia subjects and controls by using specific primary antibody in western blot by using the same total salivary protein sample that shall be preserved at -20C for future purpose. This way, we wish to come across the putative differentially expressed proteins in the schizophrenia disease in our local population cohort and that could be used as a biomarker purpose in future **(Mahmood and Yang, 2015 & Lequin, 2005).**

METHODOLOGY AT A GLANCE


Diagnosis and Executive function of schizophrenia subjects
Saliva samples from schizophrenia
Saliva sample from healthy person
Protein isolation and Quantification
Protein isolation and Quantification
Protein gel run on SDS-PAGE
Protein gel run on SDS-PAGE
Mass spectrometry (MS/MS)
Mass spectrometry (MS/MS)
Data analysis and bioinformatics
Data analysis and bioinformatics
Validation of protein (western blot)
Validation of protein (Western blot)
Comparative analysis of differentially expressed proteins towards biomarker discovery

REFERENCES

Ambatipudi, K. S., Swatkoski, S., Moresco, J. J., Tu, P. G., Coca, A., Anolik, J. H., Gucek, M., Sanz, I., Yates, J. R. & Melvin, J. E. (2012). Quantitative proteomics of parotid saliva in primary Sjögren's syndrome. *Proteomics*, *12*(19-20), 3113-3120.

Bailey, K. (1996). Brain-derived neurotrophic factor. *Chemical Factors in Neural Growth, Degeneration and Repair, (Bell C, eds)*, 203-217.

Binder, D. K., & Scharfman, H. E. (2004). Brain-derived neurotrophic factor. *Growth factors (Chur, Switzerland)*, *22*(3), 123.

Buckley, P. F., Miller, B. J., Lehrer, D. S., & Castle, D. J. (2008). Psychiatric comorbidities and schizophrenia. *Schizophrenia bulletin*, *35*(2), 383-402.

Buée, L., Bussière, T., Buée-Scherrer, V., Delacourte, A., & Hof, P. R. (2000). Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Research Reviews*, 33(1), 95-130.

Craddock, N., O'donovan, M. C., & Owen, M. J. (2005). The genetics of schizophrenia and bipolar *Journal of medical genetics*, *42*(3), 193-204.

Gelder, M. G., Juan, J., & Nancy, A. (2004). New Oxford textbook of psychiatry, Vol 1 & 2. Oxford: Oxford university press.disorder: dissecting psychosis.

Giri, K., Mehta, A., & Ambatipudi, K. (2018). In search of the altering salivary proteome in metastatic breast and ovarian cancers. *FASEB BioAdvances*, 1(3), 191-207.

Hellhammer, D. H., Wüst, S., & Kudielka, B. M. (2009). Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology*, *34*(2), 163-171.

Hu, S., Loo, J. A., & Wong, D. T. (2007). Human saliva proteome analysis and disease biomarker discovery. *Expert review of proteomics*, *4*(4), 531-538.

Iavarone, F., Melis, M., Platania, G., Cabras, T., Manconi, B., Petruzzelli, R., Cardaro, M., Siracusano, A., Faa., G., Messana, I., Zanasi, M. & Zanasi, M. (2014). Characterization of salivary proteins of schizophrenic and bipolar disorder patients by top-down proteomics. Journal of proteomics, 103, 15-22.

Ieda, M., Miyaoka, T., Kawano, K., Wake, R., Inagaki, T., & Horiguchi, J. (2012). May Salivary Alpha-Amylase Level Be a Useful Tool for Assessment of the Severity of Schizophrenia and Evaluation of Therapy.

Inagaki, T., Miyaoka, T., Okazaki, S., Yasuda, H., Kawamukai, T., Utani, E., Wake, R., Hayashid, M., Horiguchi, J. & Tsuji, S. (2010). High salivary alpha-amylase levels in patients with schizophrenia: a pilot study. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 34(4), 688-691.

Javaid, M. A., Ahmed, A. S., Durand, R., w & Tran, S. D. (2016). Saliva as a diagnostic tool for oral and systemic diseases. *Journal of oral biology and craniofacial research*, *6*(1), 67-76.

Karege, F., Perret, G., Bondolfi, G., Schwald, M., Bertschy, G., & Aubry, J. M. (2002). Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry research*, *109*(2), 143-148.

Khandaker, G. M., Zimbron, J., Dalman, C., Lewis, G., & Jones, P. B. (2012). Childhood infection and adult schizophrenia: a meta-analysis of population-based studies. *Schizophrenia research*, *139*(1-3), 161-168.

Lenzenweger, M. F., & Korfine, L. (1994). Perceptual aberrations, schizotypy, and the Wisconsin card sorting test. Schizophrenia Bulletin, 20(2), 345-357.

Lequin, R. M. (2005). Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical chemistry*, 51(12), 2415-2418.

Loganathan, S., & Murthy, R. S. (2011). Living with schizophrenia in India: gender perspectives. *Transcultural psychiatry*, 48(5), 569-584.

Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. *North American journal of medical sciences*, 4(9), 429.

Mandel, I. D. (1987). The functions of saliva. *Journal of dental research*, *66*, 623-627.

Masters, J. M., Noyce, A. J., Warner, T. T., Giovannoni, G., & Proctor, G. B. (2015). Elevated salivary protein in Parkinson's disease and salivary DJ-1 as a potential marker of disease severity. *Parkinsonism & related disorders*, *21*(10), 1251-1255.

Mercader, J. M., Fernández-Aranda, F., Gratacòs, M., Ribasés, M., Badía, A., Villarejo, C., Salano, R., Gonzalez, J. R., vallejo, J. & Estivill, X. (2008). Blood levels of brain-derived neurotrophic factor correlate with several psychopathological symptoms in anorexia nervosa patients. *Neuropsychobiology*, *56*(4), 185-190.

Moś, D. M. (2015). Saliva secretion disorder in a schizophrenic patient-a problem in dental and psychiatric treatment: a case report. *Annals of general psychiatry*, *14*(1), 14.

Nascimento, J. M., & Martins-de-Souza, D. (2015). The proteome of schizophrenia. *npj Schizophrenia*, *1*, 14003.

Neeraj, A. (2006). A short text book of psychiatry. New Delhi, Jaypee Brothers Medical Publishers (P) Ltd., 178-181.

Noto, C. S., Gadelha, A., Belangero, S. I., Smith, M. A. C., de Aguiar, B. W., Panizzuti, B., Mari, J. D. J., Gama, C.S., Bressan, R.A. & Brietzke, E. (2011). Association of biomarkers and depressive symptoms in schizophrenia. *Neuroscience letters*, *505*(3), 282-285.

Owen, M. J., Sawa, A., Mortensen, P. B. (2016). Schizophrenia. Seminar, 6736(15).

Phillips, H. S., Hains, J. M., Armanini, M., Laramee, G. R., Johnson, S. A., & Winslow, J. W. (1991). BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. *Neuron*, *7*(5), 695-702.

Picchioni, M.M. & Murray, R.M. (2007). Schizophrenia. *BMJ*, 335: 91-5.

Podzimek, S., Vondrackova, L., Duskova, J., Janatova, T., & Broukal, Z. (2016). Salivary markers for periodontal and general diseases. Disease markers.

Sabhesan, S., & Parthasarathy, S. (2005). Executive functions in schizophrenia. Indian journal of psychiatry, 47(1), 21.

Sivadasan, P., Gupta, M. K., Sathe, G. J., Balakrishnan, L., Palit, P., Gowda, H. & Sirdeshmukh, R. (2015). Data from human salivary proteome–A resource of potential biomarkers for oral cancer. Data in brief, 4, 374-378.

Rajji, T. K., Voineskos, A. N., Butters, M. A., Miranda, D., Arenovich, T., Menon, M. & Mulsant, B. H. (2013). Cognitive performance of individuals with schizophrenia across seven decades: a study using the MATRICS consensus cognitive battery. The American Journal of Geriatric Psychiatry, 21(2), 108-118.

Ray, M. T., Weickert, C. S., & Webster, M. J. (2014). Decreased BDNF and TrkB mRNA expression in multiple cortical areas of patients with schizophrenia and mood disorders. *Translational psychiatry*, *4*(5), 389.

Rehak, N. N., Cecco, S. A., & Csako, G. (2000). Biochemical composition and electrolyte balance of" unstimulated" whole human saliva. *Clinical chemistry and laboratory medicine*, *38*(4), 335-343.

Rodrigues-Amorim, D., Rivera-Baltanás, T., López, M., Spuch, C., Olivares, J. M., & Agís-Balboa, R. C. (2017). Schizophrenia: a review of potential biomarkers. *Journal of psychiatric research*, *93*, 37-49.

Ruiz, P. (2000). Comprehensive textbook of psychiatry (Vol. 1, pp. 938-950). B. J. Sadock, & V. A. Sadock (Eds.). Philadelphia: lippincott Williams & wilkins.

Saha, S., Chant, D., Welham, J., & McGrath, J. (2005). A systematic review of the prevalence of schizophrenia. PLoS medicine, 2(5), e141.

Schenkels, L. C., Veerman, E. C., & Nieuw Amerongen, A. V. (1995). Biochemical composition of human saliva in relation to other mucosal fluids. *Critical reviews in oral biology & medicine*, *6*(2), 161-175.

Slavish, D. C., Graham-Engeland, J. E., Smyth, J. M., & Engeland, C. G. (2015). Salivary markers of inflammation in response to acute stress. *Brain, behavior, and immunity*, *44*, 253-269.

Tandon, R., Gaebel, W., Barch, D. M., Bustillo, J., Gur, R. E., Heckers, S. & Van Os, J. (2013). Definition and description of schizophrenia in the DSM-5. *Schizophrenia research*, 150(1), 3-10.

Vivacqua, G., Latorre, A., Suppa, A., Nardi, M., Pietracupa, S., Mancinelli, R., Fabbrin, R., Colosimo, C., Gaudio, E. & Berardelli, A. (2016). Abnormal salivary total and oligomeric alpha-synuclein in Parkinson's disease. *PLoS One*, *11*(3), e0151156.

Whitelegge, J. P., Zabrouskov, V., Halgand, F., Souda, P., Bassilian, S., Yan, W. & Faull, K. F. (2007). Protein-sequence polymorphisms and post-translational modifications in proteins from human saliva using top-down Fourier-transform ion cyclotron resonance mass spectrometry. *International journal of mass spectrometry*, 268(2-3), 190-197.

Wig, N. N., Varma, V. K., Mattoo, S. K., Behere, P. B., Phookan, H. R., Misra, A. K., Murthy, R.S., Tripathi, B.M., Menon, D.K., Khandelwal, S.K. & Bedi, H. (1993). An incidence study of schizophrenia in India. *Indian Journal of Psychiatry*, 35(1), 11.

Yamma, K. P., & Prathibha, K. M. (2017). Methods of collection of saliva-a review. *International Journal of Oral Health Dentistry*, *3*(3), 149-153.